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13. SUPPLEMENTARY NOTES					
14. ABSTRACT HXBP-1, a basic leucine zipper protein (bZIP), is a transcription factor that belongs to the activating transcription factor/cAMP response element-binding protein (ATF/CRE) family of transcription factors and can bind to and activate the cyclic AMP response elements (CREs). In our previous studies, serial analysis of gene expression has shown that hXBP-1 may contribute to antiestrogen resistance in human breast cancer cells. In antiestrogen resistant cells CRE-regulated transcription (promoter-reporter assay) activity is increased 4-fold. Thus, the purpose of our experiments is to determine whether hXBP-1/CRE contributes to antiestrogen resistance by overexpressing hXBP-1 in cell lines that are sensitive to antiestrogens. T-47D and MCF-7 cells were transfected with hXBP-1 cDNA. Western blotting showed increased expression in transfectants; activity of this protein was confirmed in a promoter – reporter assay measuring luciferase activity as an indicator of CRE activation. Our data show that T-47D and MCF-7 cells over-expressing hXBP-1, when exposed to antiestrogens such as 4-hydroxy-Tamoxifen (TAM) and ICI 182, 780 (FAS), are able to survive the growth inhibitory effects of these antiestrogens when compared to the empty vector controls of T-47D and MCF-7 cells. Similar observations were observed when both cell types were grown in media devoid of estrogen (E2).					
15. SUBJECT TERMS HXBP-1- Human X-box binding protein; CRE- cyclic AMP response elements; E2-Estrogen					
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FOREWORD

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INTRODUCTION

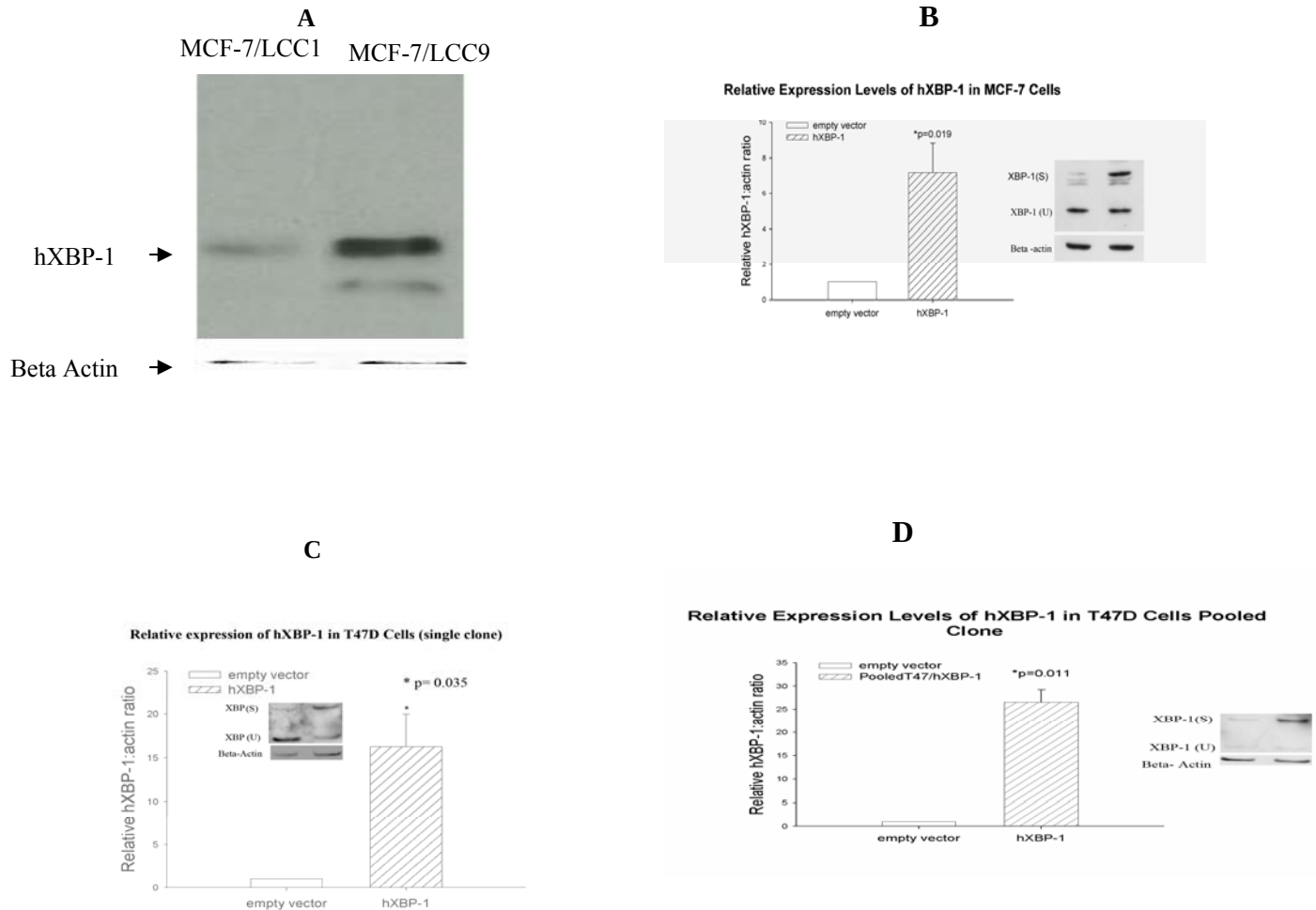
The rationale for these experiments is based on the fact that most patients with initially antiestrogen responsive breast tumors acquire an antiestrogen resistant phenotype (1). Although several possible mechanisms such as mutations in the estrogen receptor (ER) and loss of ER expression have been associated with antiestrogen resistance, other possible mechanisms that can contribute include Tamoxifen stimulated resistance mechanisms (1). Tamoxifen (ICI 46,477) is the most widely used antiestrogen for breast cancer treatment. Faslodex (ICI 182,780) is another antiestrogen therapy used for breast cancer treatment (2). It is effective in patients who have acquired resistance to Tamoxifen treatment. In these experiments, MCF-7 and T-47D cells stably transfected with pcDNA 3.1/hXBP-1 will be used to determine resistance to these antiestrogens when hXBP-1 is over-expressed.

We hypothesize that the over expression of hXBP-1 in cell lines that are sensitive to antiestrogens will confer estrogen independence and antiestrogen resistance. Stable transfection with the pcDNA/hXBP-1 construct in both MCF-7 and T-47D breast cancer cells was used to create MCF-7/hXBP-1 and T-47D/hXBP-1 cells respectively. Both of the parent cell lines are sensitive to antiestrogens such as 4-OH Tamoxifen (TAM) or Faslodex (FAS). Overexpression of hXBP-1 produced the expected increase in CRE transcriptional activation, which was measured by promoter- reporter assays. Transfected cells treated with antiestrogens have a better survival rate *in vitro* when compared to the non-transfected MCF7 cells; hence, hXBP-1 overexpression conferred resistance to these antiestrogens. Resistance to antiestrogens may reflect changes in proliferation and/or effects on apoptosis. Western blot analysis shows an increase in Bcl-2 expression after antiestrogen treatment MCF-7/hXBP-1 cells but not in T-47D/hXBP-1 over-expressing cells – despite Bcl-2 gene or protein expression being induced by estrogens in wild-type T47D cells (3). Thus, the different cellular contexts of MCF-7 and T-47D cells may cause these cell lines to use different signals down-stream from hXBP-1 to confer resistance.

Since this is a final report, we show all the major findings from the thesis research. I am currently preparing manuscripts for submission and will defend my thesis within the next few months. The training I have received, and the exciting work I have completed in breast cancer research, would not have been possible without this DOD BCRP Predoctoral Fellowship Award.

Task 1. To determine if sensitive cells can become resistant to antiestrogens Tamoxifen and Faslodex by the over-expression of hXBP-1 in breast cancer cells (Months 1-24)

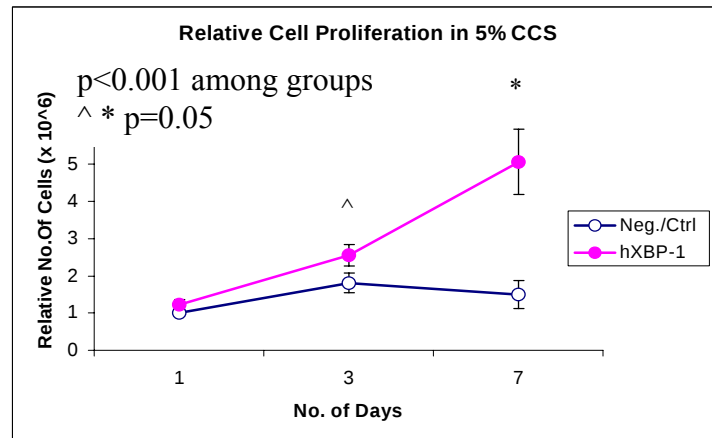
Figure 1. The differential levels of hXBP-1 spliced (S) expression in MCF-7 or T-47D cells. A, basal expression levels of hXBP-1 in antiestrogen sensitive cells (MCF-7/LCC1) and antiestrogen resistant cells (MCF-7/LCC9). B-D, over-expression of hXBP-1 after stable transfection.



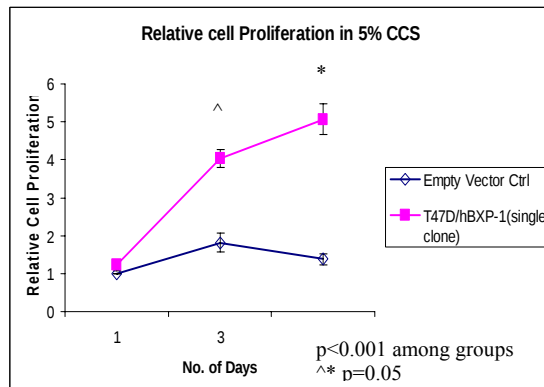
For western blot analysis 20 ug of total protein was isolated from hXBP-1 transfected MCF7 or T47D cells and resolved by electrophoresis using NuPage 10% Bis-Tris gels and blotted onto nitrocellulose membranes. After blocking, the nitrocellulose membrane was washed and incubated with rabbit polyclonal hXBP-1 primary antibody (1:200) (Santa Cruz). Beta actin primary antibody (1:5000) was used to compare loading. Antigen-antibody complexes were then visualized with the ECL detection system (Amersham) and exposed to film. The p values were determined by the paired-t test (n=3 independent experiments).

Figure 2. Growth efficiency is higher in MCF7/hXBP-1 and T47D/hXBP-1 cells in the absence of estrogens.

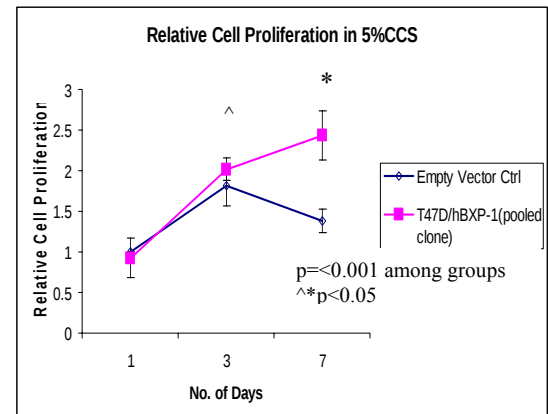
A. MCF7/hXBP-1 Cells (single clone)



B. T47D/hXBP-1 Cells (single clone)



C. T47D/hXBP-1 Cells (pooled clone)



MCF7 or T47D cells over expressing hXBP-1 and their empty vector controls were allowed to grow in IMEM without phenol red supplemented with 5% charcoal stripped calf serum (CCS) for 72 hours. After growth devoid of estrogen, the cells were plated at a density of 3.5×10^4 cells/well into 12 well plastic tissue culture plates and allowed to grow. On days 1, 3 and 7 post plating the cells were then trypsinized, resuspended in PBS and counted using a Beckman coulter counter (Beckman Coulter Corp., Fullerton, CA) to determine growth efficiency. p-values were obtained using the Student-Newman-Keuls Method; n=3 independent experiments.

Figure 3. Cell proliferation of MCF7 and T47D cells stably transfected with hXBP-1 in 5% FBS supplemented with 4-OH TAM or FAS is increased when compared to the empty vector control. Cells were harvested and counted as above. p values obtained by One way ANOVA and Tukey test; n=3 independent experiments.

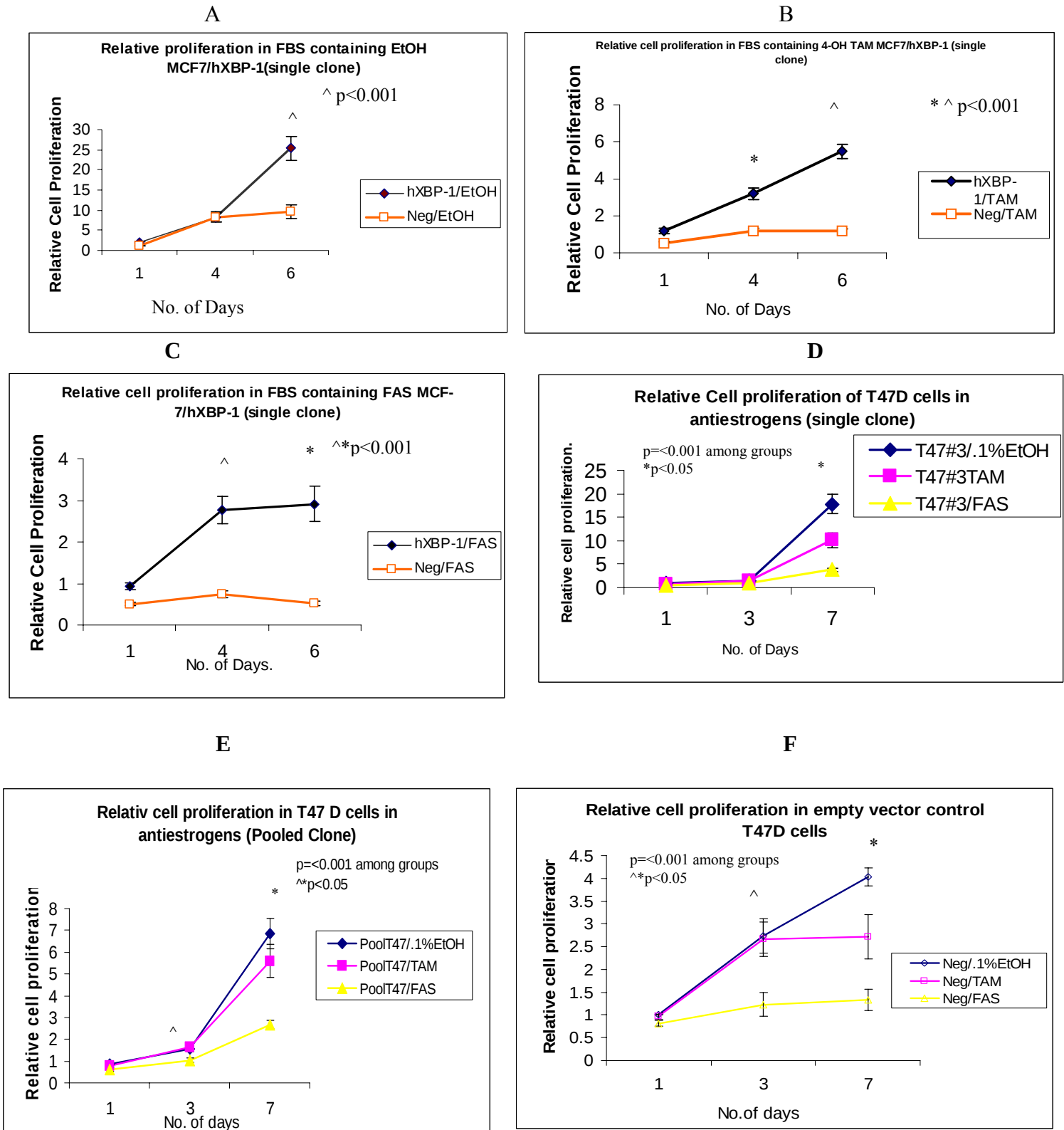
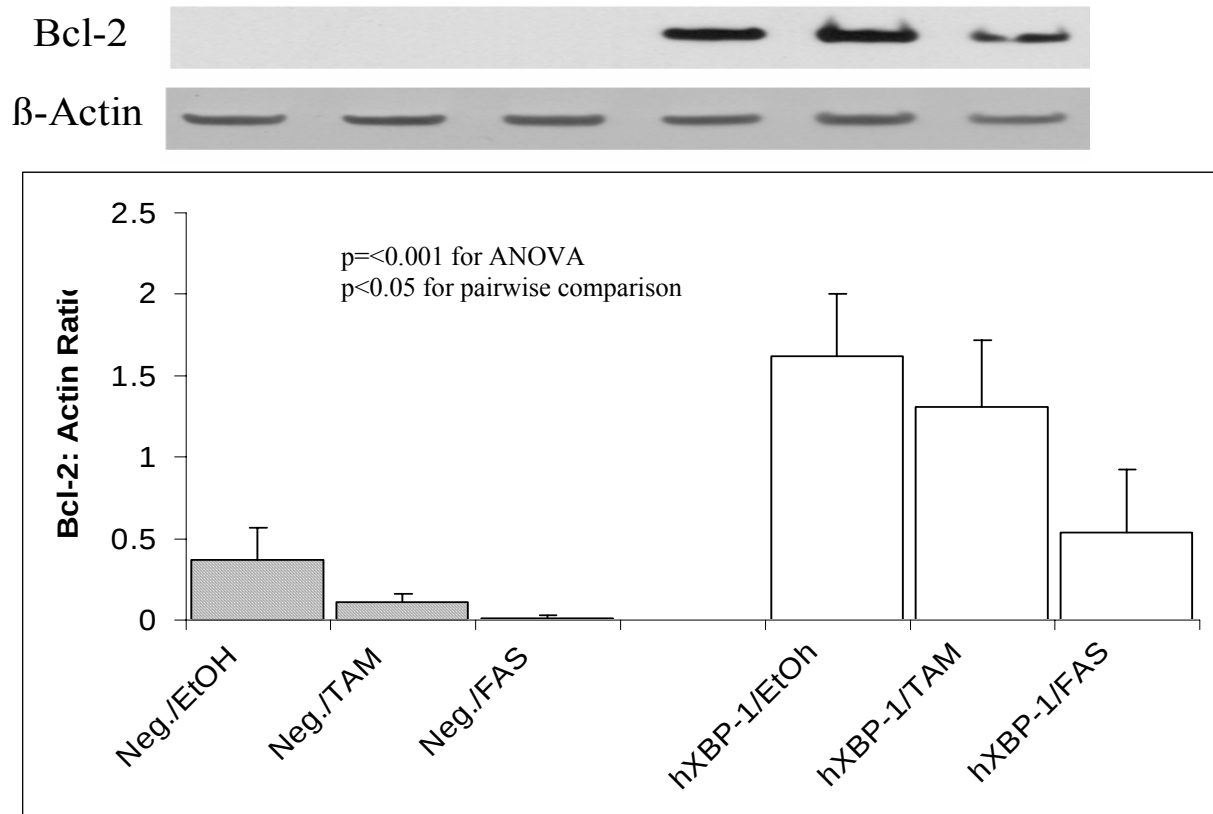
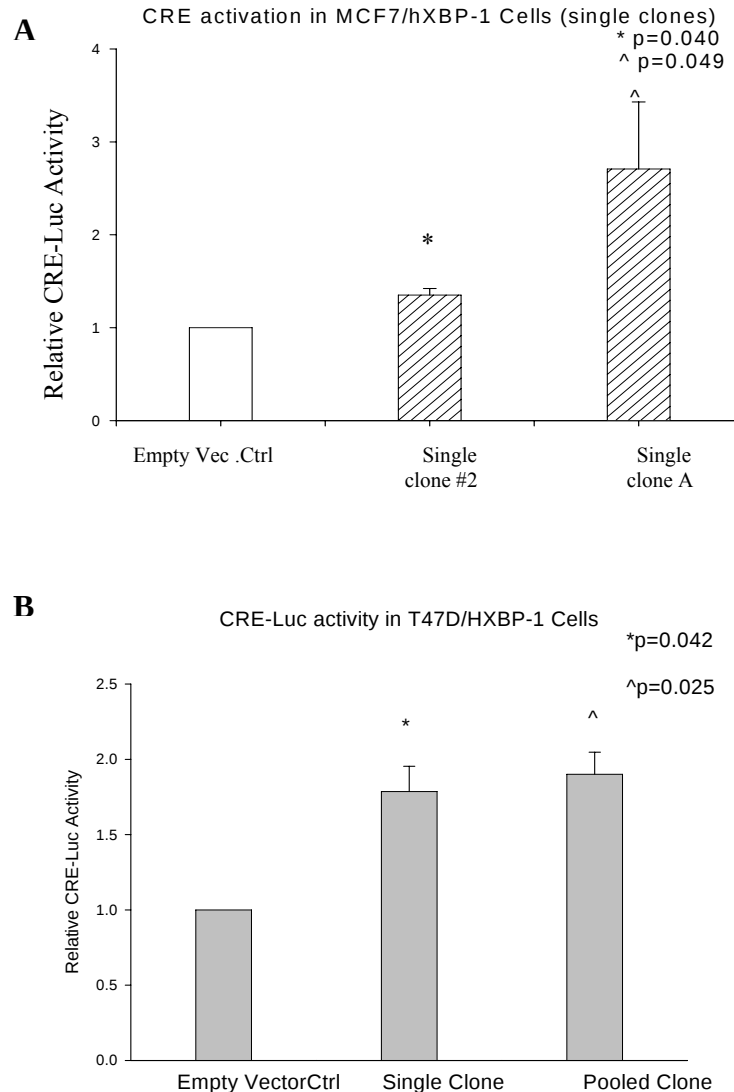


Figure 4. Bcl-2 expression is increased in MCF-7/hXBP-1 cells following antiestrogen treatment.



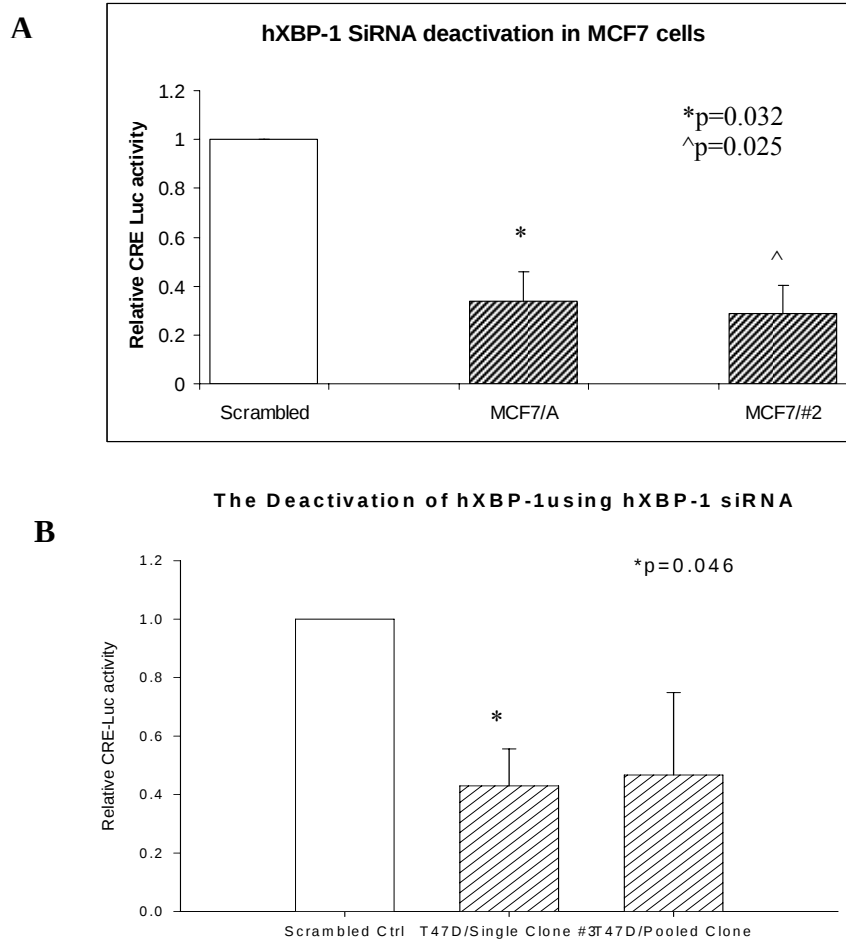
For western blot analysis 20 ug of total protein was isolated from hXBP-1 transfected MCF-7 cells and resolved by electrophoresis using NuPage 10% Bis-Tris gels and blotted onto nitrocellulose membranes. After blocking, the nitrocellulose membrane was washed and incubated with mouse monoclonal Bcl-2 primary antibody (1:200) (Stressgen). Beta actin primary antibody (1:5000) was used to compare loading. Antigen-antibody complexes were then visualized with the ECL detection system (Amersham) and exposed to film. The p values were determined by ANOVA; n=3 independent experiments. The baseline signals for Bcl2, which are variable and not always visible, are usually detected in the digitized data by the software.

Figure 5. There is increase in CRE transcriptional activity in MCF-7/hXBP-1 and T-47D/hXBP-1 when compared to their empty vector controls; n=3 independent experiments.



About 3.5×10^4 cells/well were plated in 12 well plates and allowed to grow for 24 hours prior transfection. Cells were cotransfected with 0.4 ug of CRE- luciferase plasmid. To account for possible differences in transfection efficiency, 0.1 ug of a plasmid containing the *Renilla* luciferase gene was also cotransfected into the cells. Both plasmid DNAs were added to a mix of Fugene 6 (Roche) and serum free IMEM after their incubation at room temperature for 5 mins., after which an additional incubation of 15 mins was carried out at room temperature. The transfection mix was added to each well and incubated at 37°C for 24 hours. Cells were maintained in media containing serum. After transfection, fresh media was added to each well for 24 hours. Cells were then lysed and the activation of the CRE-luciferase construct was measured using the Dual Luciferase Assay Kit (Promega). Luminescence was quantitated using a Lumat LB 9501 luminator (EG&G Berthold, Bundoora VIC, Australia). P-value was obtained using the Students t-test; n=3 independent experiments.

Figure 6. CRE-Luc activity is decreased by hXBP-1 siRNA.

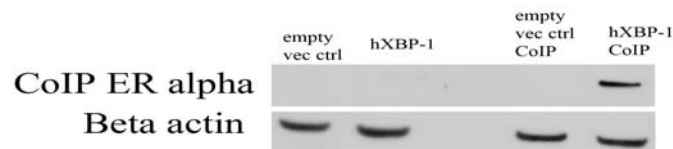


Approximately $0.4-1.6 \times 10^5$ were seeded per well in a 24-well plate in 500ul of red IMEM supplemented with 5% FBS and 200-800 $\mu\text{g/ml}$ of G418 and allowed to grow over night at 37°C in 95% air and 5% CO_2 prior to transfection. A final concentration of 5 nM hXBP-1 siRNA (Qiagen) was used for transfection. Cells were kept in transfection media for 72 hrs and incubated under conditions indicated above to allow gene silencing. A scrambled sequence was used as a positive control in all clone types for CRE-luc activity normalization. After 72hrs cells were cotransfected with 0.4 μg of CRE- luciferase plasmid. To account for possible differences in transfection efficiency, 0.1 μg of a plasmid containing the *Renilla* luciferase gene was also cotransfected into the cells. Both plasmid DNAs were added to a mix of Eugene 6 (Roche) and serum free IMEM after their incubation at room temperature for 5 mins, after which an additional incubation of 15 mins. was carried out at room temperature. The transfection mix was added to each well and incubated at 37°C for 24 hours. Cells were maintained in media containing serum. After transfection, fresh media was added to each well for 24 hours. Cells were then lysed and the activation of the CRE-luciferase construct was measured using the Dual Luciferase Assay Kit (Promega). Luminescence was quantitated using a Lumat LB 9501 luminator (EG&G Berthold, Bundoora VIC, Australia). p-values were obtained using the paired t-test; n=3 independent experiments. In the data the scrambled control is representative for all clones after normalization.

Figure 7. ER alpha co-immunoprecipitates with hXBP-1 in MCF-7 and T47-D cells.

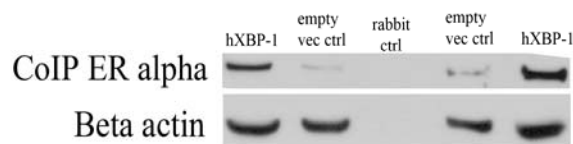
CoIP for MCF7/hXBP-1 (single clone #2)

A



CoIP ERalpha MCF-7/hXBP-1 (single clone A)

B



CoIP ER alpha in T47D/hXBB-1 (pooled clone)

C

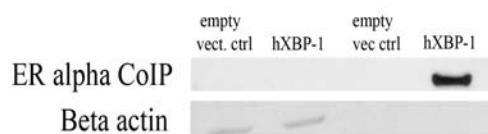
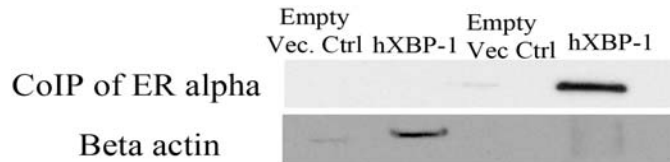


Figure 7 cont'd. ER alpha co-immunoprecipitates with hXBP-1 in MCF-7 and T47-D cells.

CoIP of ER alpha in T47D single clone

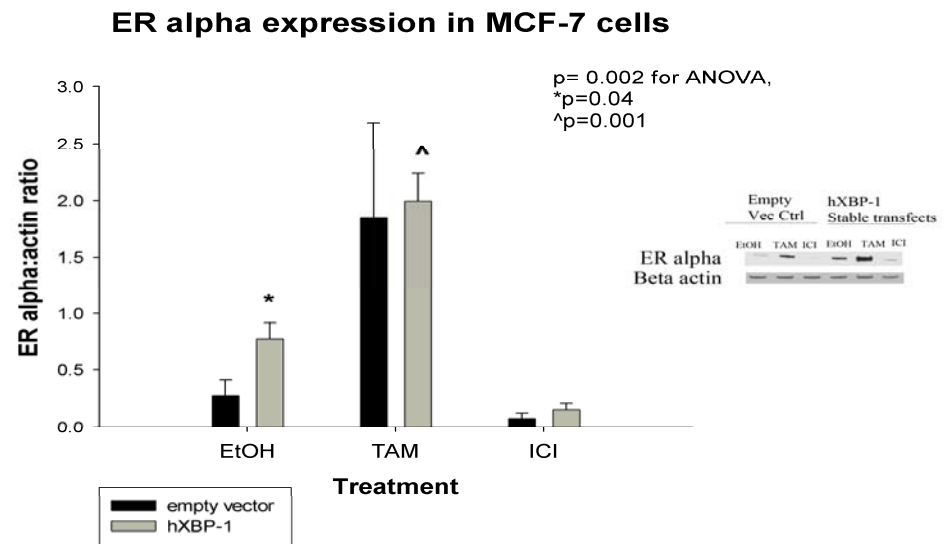
D



Approximately 1µg/µl of cell lysate will be added to an immunoprecipitating antibody (a rabbit polyclonal antibody to hXBP-1) immunoprecipitation will occur overnight at 4⁰ C with rocking. Following a 2 hour incubation (4⁰C with rocking) with protein A-Sepharose beads, samples will be centrifuged to precipitate the immune complexes with the beads; the beads will be washed 2 times with RIPA buffer followed by 2 washes with Tris-Saline (150mM, 50mM Tris Base pH 7.5) and resuspended in 30-50 µl of 2X sample buffer, and boiled for 5 mins. Immunoprecipitation will be analyzed by electrophoresis using NuPage 10 % Bis-Tris polyacrylamide gels. Left hand side two lanes represent lysates, right hand side lanes represent the coimmunoprecipitations.

Figure 8. hXBP-1 over expression increases ER alpha expression

A



B Relative Expression Levels of ER alpha in T47D Cells (pooled clone)

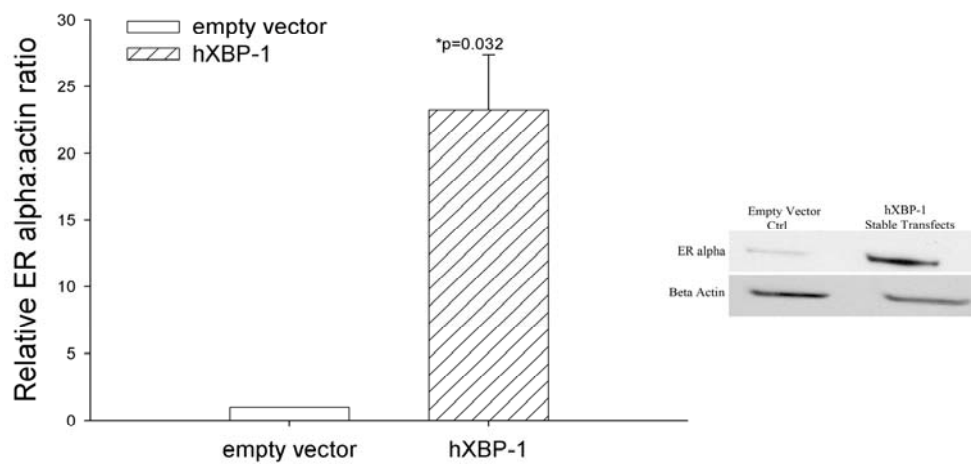
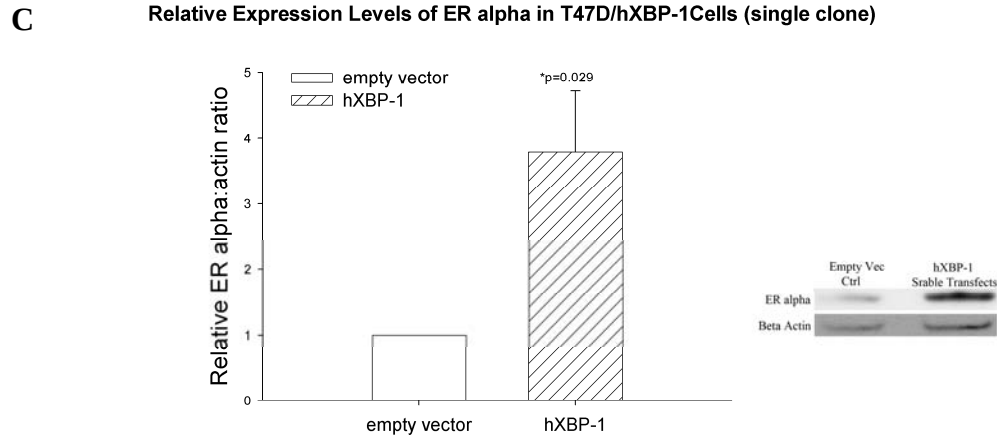


Figure 8 cont'd. hXBP-1 over expression increases ER alpha expression



For western blot analysis 20 ug of total protein was isolated from hXBP-1 transfected MCF7 cells and resolved by electrophoresis using NuPage 10% Bis-Tris gels and blotted onto nitrocellulose membranes. After blocking, the nitrocellulose membrane was washed and incubated with mouse monoclonal ER alpha primary antibody (1:200) (Vector Lab; Burlingame, California). Beta actin primary antibody (1:5000) was used to compare loading. Antigen-antibody complexes were then visualized with the ECL detection system (Amersham) and exposed to film. The p values were determined by the paired t-test; n=4 independent experiments.

KEY RESEARCH ACCOMPLISHMENTS

The goal of task 1 was to show:

- Greater survival in media devoid of estrogens and antiestrogen containing media of MCF-7 and T-47D cells stably transfected with pCDNA 3.1 hXBP-1.

MCF-7 and T-47D cells overexpressing hXBP-1 have a significantly increased growth rate when compared to empty vector control cells in media devoid of estrogen (5%CCS) through day #3 to #7 (**Fig. 2**; * $p < 0.05$). Furthermore, our data show that MCF-7 and T47-D cells over-expressing hXBP-1, when exposed to antiestrogens such as 4-hydroxy Tamoxifen (TAM) and ICI 182,780 (FAS), exhibit reduced sensitivity to these antiestrogens, **Fig 3**.

- Measure CRE transcriptional activity to confirm hXBP-1 activity.

Fig. 5 shows a significant increase in relative CRE activity in cells overexpressing hXBP-1 when compared to empty vector control cells.

Fig. 6 shows that hXBP-1 siRNA expression decreases CRE transcriptional activity.

- The mechanism of antiestrogen resistance may involve an anti-apoptotic pathway.

Bcl-2 expression is increased in MCF-7/hXBP-1 after exposure to antiestrogens.

Fig. 7 and 8 western blot analysis and co-immunoprecipitation assays show that in MCF-7/hXBP-1 and T-47D/hXBP-1 cells there is increased estrogen receptor alpha expression and estrogen receptor interaction respectively.

REPORTABLE OUTCOMES (entire funding period)

- ✓ Zhu Y, Singh B, Hewitt S, Liu A, **Gomez B**, Wang A and Clarke R. “Expression patterns among interferon regulatory factor (IRF-1), human X-Box Binding Protein-1 (hXBP-1), Nuclear Factor Kappa B (NFκB), Nucleophosmin (NPM), Estrogen receptor-alpha (ERα), and progesterone receptor proteins in breast cancer tissue microarrays”. *International Journal of Oncology*, in press, 2005.

- ✓ **Gomez, B.P.**, Riggins, R., Zhu, Y., Zwart A. & Clarke, R. "Human X Box Binding Protein in Antiestrogen Resistance". *Department of Defense Breast Cancer Research Program Meeting*, 4th Era of Hope, Philadelphia, Pennsylvania, 2005 (abstract).
- ✓ Clarke, R., Riggins, R.B., Bouker, K.B., Nehra, R., **Gomez, B.** & Zwart, A. "Molecular mechanisms of endocrine resistance in breast cancer." *Anticancer Res*, 24: 3455-3456, 2004 (abstract).
- ✓ Riggins, R.B., **Gomez, B.**, Zwart, A.L. & Clarke, R. "NFκB signaling promotes antiestrogen resistance in MCF-7 breast cancer cells." *Proc 95th Am Assoc Cancer Res*; Abstr 3498, 2004 (abstract).
- ✓ **Gomez, B.P.**, Riggins, R., Zhu, Y., Zwart A. & Clarke, R. "Human X-box binding protein in antiestrogen resistance." *Proc 95th Am Assoc Cancer Res*; Abstr 1213, 2004 (abstract).
- ✓ Clarke, R., Liu, C.M., Bouker, K.B., Gu, Z., Lee, R.Y., Zhu, Y., Skaar, T.C., **Gomez, B.P.**, Brien, K., Wang, Y., Hilakivi-Clarke, L. "Antiestrogen resistance in breast cancer and the role of the estrogen receptor". *Oncogene*, 22: 7316-7339, 2003.
- ✓ Zhu, Y., Bouker, K., Skaar, T., **Gomez, B.**, Hewitt, S., Singh, B., Liu, A. & Clarke, R. "High throughput tissue microarray study of antiestrogen related genes in human breast cancers." *Proc 85th Annual Meeting Endocrine Society* 140, 2003 (abstract).
- ✓ Zhu, Y., Bouker, K., Skaar, T., Zwart, A., **Gomez, B.**, Hewitt, S., Singh, B., Liu, A. & Clarke, R. "High throughput tissue microarray assessment of expressions of progression-related genes - NFκB, nucleophosmin, X-box binding protein-1 and IRF-1 in breast cancer." *Proc Am Assoc Cancer Res* 43: 762, 2002 (abstract).

CONCLUSIONS

1. Over-expression of hXBP-1 in cells that are estrogen dependent for growth confers estrogen independent growth.
2. Our data with ICI 182,780 (FAS) and 4-OH Tamoxifen (TAM) suggest that increased hXBP-1 expression reduces sensitivity to these antiestrogens.
3. hXBP-1 may be a key factor that is involved in acquired resistance to TAM and FAS in breast cancer cells.

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3. Dong, L., Wang, W., Stoner, M., Reed, J.C., Harigais, M., Samudio, I., Kladde M. P., Vyhldal, C., Safe S. Mechanisms of transcription Activation of bcl-2 Gene Expression by 17 β -Estradiol in Breast Cancer Cells. *The Journal of Biological Chemistry*, 274: 32099-32107, 1999.